Isolation and characterisation of mutants from the halotolerant yeast *Pichia sorbitophila* defective in H^+/glycerol symport activity

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1. ABSTRACT

Picha sorbitophila, a yeast species highly resistant regarding osmostress in general and to salt stress in particular, was subjected to a mutagenesis strategy in order to obtain mutants deficient in the glycerol active uptake previously described. Density centrifugation was used for enrichment of NaCl sensitive mutants in either glucose or glycerol media. Several phenotypic classes of mutants were identified, to which physiological tests were applied concerning the activity of the symporter, its accumulation capacity and the detection of the activity of glycerol pathway specific enzymes. From these, two mutant strains were selected, presenting a clearly deficient phenotype on H^+/glycerol symport activity.

2. INTRODUCTION

The so-called osmotolerant yeasts, and among them the halotolerant ones, constitute an heterogeneous group of microorganisms, sharing the common capacity of resisting low a_W environments [1,2]. Up to the moment, the main mechanism that has been identified in yeasts as a biochemical response to osmotic stress, in particular caused by high quantities of sodium chloride, is the production and intracellular accumulation of low molecular weight molecules that function as osmoprotectants. In yeasts this role is mainly played by glycerol, followed by arabitol and other polyhydroxy alcohols (polyols) to lower extent [3,4,5,6,7]. Taking into consideration the fact that polyols are liposoluble molecules, leaking through the plasma membrane, retention has thus to be an active response from the cell. In two taxonomically unrelated yeast species, *Debaryomyces hansenii* and *Pichia sorbitophila,* two different types of constitutive active transport systems for glycerol have already been characterised. In the first case, a sodium-glycerol co-transport [8] and in the second case, a proton-glycerol symport [9]. Besides, the existence of another active glycerol transporter has been demonstrated in *Zygosaccharomyces rouxii* [10]. As a first step, in order to enable future studies of the Molecular Biology of the glycerol transport, the work here presented corresponds to the generation of mutants from *Pichia sorbitophila* deficient in active glycerol uptake and/or retention and thus affected in their osmoregulation capacity.

3. MATERIALS AND METHODS

3.1. Yeast strains media and growth conditions

Pichia sorbitophila CBS 7064 wild type strain, was maintained in YEPDA at 25°C and mutant strains were kept frozen at -70°C in glycerol 30%w/v. Growth was performed and monitored as described before [9].

3.2. Sporulation media and spore manipulation

Sporulation of *P. sorbitophila* was assayed in classic sporulation media: acetate, 5% malt, Gorodkowa, "Corn meal", PDA, YM and YEPSA; as well as in YEPDA and YEPGA containing up to 4M NaCl. Asci walls were digested with β-glucuronidase/arylsulfatase (water solution from Boehringer Mannheim) at room temperature [11]. Isolation of ascospores was performed using home made micro manipulation needles and a Leitz micro manipulator adapted to a Olympus CK2 inverted microscope. Each ascospore isolated from a set of six tetrads was cultivated separately in YEPDA at 28°C and kept frozen at -70°C as mentioned above.

3.3. Mutagenesis

P. sorbitophila was grown in liquid mineral medium [9] up to mid-exponential phase (A_{640nm} $0.8 \approx 4.6x10^7$ cells/ml). 5ml culture were harvested by centrifugation, washed twice in cold distilled water and ressuspended in 5ml sterile 50mM phosphate buffer pH 7. Mutagenesis, was attained incubating with 20μg/ml N-methyl-N'-nitro-N-nitrosoguanidine (NG) (Sigma), final concentration, at 28°C with magnetic stirring for 14 min. This treatment gave origin to a 10% survival of the population. NG action was stopped adding 5ml sodium thiosulfate 10% (w/v). The mutagenised culture was centrifuged, washed twice as before and transferred to 50ml YEPD. Recovery was allowed incubating the cells for 36h at 28°C.

3.4. Gradient centrifugation

To select cell populations according to their density, we used a method described earlier [12] with some modifications. Density gradients were prepared using Percoll (Pharmacia) and sorbitol 4,25M in Beckman Ultra Clear™ centrifuge tubes. A 4ml sample of cell culture was applied directly on top of the gradient and centrifuged for 30 min. at 4°C at 19000 g in a Beckman XL-90

Ultracentrifuge, using a Swinging Bucket Rotor SW 28. For mutant selection, bottom samples (3ml) were collected.

3.5. NaCl maximum tolerance experiments

Resistance to salt was monitored in YEPDA and YEPGA, supplemented with different NaCl concentrations. Mutant phenotypes were searched by replica plating and confirmed using the yeast suspension drop method. No previous adaptation to stress media was performed.

3.6. Measurement of initial uptake rates of glycerol and protons and of glycerol accumulation ratios

Glucose growing cells in minimal medium [9] were harvested in mid-exponential phase (A_{640nm} 0.8) by centrifugation, washed twice and resuspended to a final concentration of about 30 mg (dry wt) ml^{-1} in ice-cold distilled water. Initial rates of proton uptake upon glycerol addition, initial uptake rates of [^{3}H]glycerol, as well as maximum accumulation capacity against gradient of [^{3}H]glycerol, were estimated using the methodologies described previously [8,9]. For every case, [^{14}C]glycerol was used to confirm results.

Membrane H^+/ATPase activity was detected using the same methodology mentioned above to measure proton uptake. Extracellular acidification velocity was measured in the linear part of the acidification curve obtained after cell energisation with a pulse of 10mM glucose.

3.7. Enzyme activity in vitro *assays*

Glycerol 3-P phosphatase and glycerol 3-P dehydrogenase (NAD^+) were assayed according to the methods described in, respectively, [13] and [14].

4. RESULTS

4.1. Sporulation assays and putative haploid phase stabilisation prior to mutagenesis

Conjugation, in *P. sorbitophila* , has been described to happen between independent cells or mother and bud [15], results from which one could infer homothalism. In the absence of further documentation, we tried to confirm these results assaying sporulation in a set of conventional media (Materials and Methods). Sporulation was obtained in Malt 5%, YEPGA, PDA, "Corn Meal", YM

and YEPSA. Homothalism was confirmed crossing clones from ascospore with each other. Sporulation was obtained when each of these pure cultures germinated alone in Malt 5%. In the attempt to define how stable haploid phase could be in media that did not induce sporulation, cultures from each ascospore were inoculated in every solid and liquid medium to be used in further mutant isolation, selection and characterisation: YEPDA, YEPGA and mineral medium with 2% (w/v) glucose w/ or w/o agar 2% (w/v), w/ or w/o NaCl up to 4M. Cultures were surveyed intensively until 4 weeks incubation time. Neither zygotes or mother-bud conjugation forms, nor asci or ascospores, were ever detected, indicating there was no induction of sporulation in any of these incubation and growth conditions, even under severe salt stress, as long as the carbon source was not glycerol. Mother-bud conjugation forms, asci and ascospores were observed in YEPGA cultures independently of the presence and concentration of NaCl. Each of the YEPDA cultures was then transferred to Malt 5% for control, where, again, sporulation was observed. One ascospore culture was then chosen to perform mutagenesis.

4.2. NaCl induced density changes as an enrichment strategy

In order to establish an enrichment procedure for *P. sorbitophila*, based on cellular density changes due to glycerol intracellular accumulation in response to sodium stress, like has been described for *Debaryomyces hansenii* [12], cells were grown in mineral medium with glucose 2% (w/v) in the absence and in the presence of 1M and 2M NaCl. Cells were collected in mid-exponential growth phase and submitted to density gradient centrifugation, directly and, in alternative, after washing twice in ice-cold distilled water. Cells banded on top of the gradient when growth was performed in the presence of NaCl and intracellular glycerol concentration was high. As long as growth was performed in the absence of salt stress, or when the cells were washed, they banded on the bottom of the gradient, forming a more or less dense pellet. Alternatively, and in a similar way to what has been published for *D. hansenii* [12], to obtain the same decrease in cell density, it was enough to incubate them in NaCl supplemented growth medium for at least 4 hours. It was thus anticipated that mutants defective on glycerol production or retention, having either a leaky membrane or the glycerol active transport system affected, or not present at all, would band on the bottom of the gradient. Mutagenised culture, after recover, was incubated in mineral medium

with glucose 2%(w/v) and 1M NaCl for 4 hours at 28°C and centrifuged. Most of the cells, as expected, banded on top of the gradient. The bottom fraction was collected for mutant selection.

4.3. Phenotypic selection of mutant strains with wild type halotolerance pattern changed

Phenotypic selection was performed with two sets of different selective media, having either glucose or glycerol as carbon and energy sources, varying the intensity of salt stress with NaCl concentrations up to 3M. Mutants were found more sensitive to stress in either glucose or glycerol, or else, regardless to the carbon source used (Table 1). All of them were able to metabolise glycerol. Their distinctive feature was that resistance to NaCl, comparing to the wild type, was not completely eliminated but just decreased more or less severely.

4.4. Physiological characterisation relating glycerol active transport system

Each mutant strain thus obtained was then submitted to a series of tests to evaluate to which extent H^+/glycerol symport had been affected. Cells were grown in glucose medium and harvested by centrifugation in mid-exponential phase. Cell suspensions were then assayed in what regards alkalinisation induced by glycerol (Table 2) and labelled glycerol uptake, in the absence as in the presence of 1M NaCl in the assay mixture (Table 3). This survey was complemented testing the maximum labelled glycerol intracellular accumulation against gradient (Table 3). In parallel, the activity of membrane proton ATPase was verified, also in the absence and in the presence of 1M NaCl in the assay mixture (Table 2). In one mutant strain alone, 3.42 (Class VII), proton extrusion after glucose energisation was not present at all, making it impossible to determine whether H^+/glycerol symport was also affected or not because of the consequent lack of membrane potential indispensable to allow the activity of secondary active transport. Iterative procedures were applied to two mutant strains alone, 2.4 (Class II) and 2.18 (Class V), which presented kinetic parameters for active uptake and simple diffusion still measurable. After iteration, the active uptake parameters disappeared, remaining diffusion constants comparable to the ones published for wild type strain [9] (Table 4). In order to determine if the absence of uptake could be due to a secondary effect caused by multiple mutations eventually affecting the activity of any of the enzymes of glycerol pathway, and since glycerol consumption was clearly possible, the same strains were selected to perform *in vitro* assays of glycerol production specific enzymes. Glycerol 3-P phosphatase and glycerol 3-P

dehydrogenase (NAD^+) activities determined in either mutant strain were identical to the wild type, respectively 16.4±3.8 (6) and 51.9±6.3 (9) mU/mg protein.

5. DISCUSSION

Results obtained with all mutant strains assayed in what regards halotolerance pattern of resistance allowed us to create different mutant classes which maintained, with almost no exceptions, a degree of similarity in all the physiological assays. The first physiological assays, performed to every mutant strain, concerned proton alkalinisation due to H^+/glycerol symport activity and H^+/ATPase activity. All strains presented deficient or non-existing proton uptake upon glycerol addition. On the other hand, in just one case a clear phenotype of H^+/ATPase activity deficiency was visible - 3.42 from Class VII. In all other strains a more or less active proton pump could be detected. This reassured us in what concerns the maintenance of proton motive force, and thus the true malfunctioning of the glycerol symporter. To execute radiolabelled glycerol assays, one strain of each mutant class was chosen as representative. Each of them was thus subjected to assays of glycerol uptake to determine the kinetic constants of the active transport, as well as the simple diffusion constant. This one should, in case of presenting a higher value than the wild type, give some indication of the cell membrane permeability and allow us to detect increased leakiness. This was the case of almost all strains, generally speaking. Nevertheless, kinetic parameters were still measurable in some of the strains, like 2.4 (II) and 2.18 (V), in which cases iteration procedures confirmed those to be artefacts. Accumulation capacity of the active glycerol uptake confirmed these results, presenting an in/out glycerol ratio close to or less than equilibrium, just like it is expected for a compound crossing the membrane by other than a mediated active transport system. From all the results presented, mutant strains belonging to the Classes I, II, IV, V and VI were considered to present a phenotype more close to what we could expect from an active glycerol uptake deficient mutant, from which we stress 2.4 (II) and 2.18 (V) strains. These, besides presenting a clear deficiency in respect to active glycerol uptake, are not affected in the activity of the specific enzymes of glycerol metabolic pathway.

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